in vivo Evaluation of Bone Regeneration by TAT-BMP-2 and Hydrogel Composite

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감사의 글

馬行處 牛亦去 라. '말 가는 곳에 소는 못 가랴' 는 마음으로 시작하였지만, 말 가는 길 틀리고 소 걷는 길 다름을 깊이 깨우친 한 시절이었습니다. 쭉 뻗은 신작로로 갈기 휘날리며 달리는 馬群 옆에 논두렁 밭두렁을 질척대며 뚜벅뚜벅 걷는 늙은 소의 워낭소리를 메아리처럼 가슴으로 듣곤 하였습니다.

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순한 눈매와 따뜻한 체온으로 안겼다가 외로운 고통 속에서 힘들어 하며 저 세상으로 떠난 임상연구동의 가여운 인연들. 서방정토 아미타 극락정토에 왕생하시어 좋은 몸 받으시고 부처님 시봉 잘 하시길 발원합니다.

30년 긴 세월 동안, 그 많던 사연 속에서 묵묵히 함께해주고 내편이 되어준 내 아내에게 표현할 수 없는 깊은 사랑으로 이 영광을 돌립니다.

어느 한 순간도 내려놓지 않으시고 그 많던 어려움과 위험 속에서 늘 지켜주시고 무한한 자비로 오늘이 있게 해주신 제불보살님께 이 모든 공덕 회향해 올립니다.

나무마하반야바라밀

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in vivo Evaluation of Bone Regeneration by TAT-BMP-2 and Hydrogel Composite

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(Directed by Professor Jong In Yook, D.D.S., Ph.D.)

Although the need for bone regeneration is gradually increasing due to aging, alveolar bone loss, and osteoporosis, clinical modality is not yet definite. Exogenous bone morphogenetic protein (BMP) should have sufficient bioactivity and be in harmony with carriers by controlled release of biological factors with mechanical support. Recombinant human BMP-2 (rhBMP-2) are currently used in clinics, but their efficacy remains obscure.

Contrary to rhBMP-2, inactive polypeptide TAT-BMP-2 fusion protein can be successfully transduced into cells in concentration- and time-dependent manners independent to BMP receptors. Hydrogel, which is made of hyaluronic acid, one of the major components of human connective tissue, shows biocompatibility and releases containing materials in a controlled manner with degradation. This study aims to evaluate

bone regeneration radiologically and histologically with the femurs of beagles by combining TAT-BMP-2 with fibrin, hydrogel, and PEEK. The results are as follows:

1. TAT-BMP-2 fibrin composite induced superior bone regeneration than the fibrin-only applied subject under isolated condition by PEEK, but statistically not significant.
2. The application of hydrogel alone induced partial bone regeneration.
3. TAT-BMP-2 hydrogel composite caused superior bone regeneration than the application of hydrogel alone in area, volume analysis ($p<0.05$).
4. TAT-BMP-2 hydrogel composite only induced regeneration of continuity of the outer cortex and showed a more matured, dense structure than the application of hydrogel alone.
5. TAT-BMP-2 hydrogel composite decreased dosage for *in vivo* bone regeneration by 1/1000 of the conventional dosage of rhBMP-2.

These results indicate that TAT-BMP-2 hydrogel composite induced effective *in vivo* bone regeneration by highly efficient protein synthesis through inactive polypeptide TAT-BMP-2 as well as controlled protein releasing through hydrogel.

Key words: PTD (Protein Transduction Domian), HIV-1 TAT, BMP (Bone Morphogenetic Protein), hydrogel

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I. INTRODUCTION

Attempts to induce *de novo* bone formation are among the most interesting experiments in clinics. Although the need for bone repair and replacement is gradually increasing due to aging, trauma, and tumors, this field still remains a significant challenge. Tissue engineering for bone regeneration can be described as a combination of biological, chemical, and engineering principles toward the repair, restoration, and replacement of tissues using cells, scaffolds, and biologic factors alone or in harmonious combination (Laurencin et al., 2006).

For successful bone regeneration, osteoprogenitor cells should be differentiated into osteoblast by several growth factors, of which bone morphogenetic protein (BMP) plays a major role(Li and Wozney, 2001). BMPs are members of the transforming growth factor-

beta superfamily and elicit cellular effects through the induction of heteromeric complexes of type I and type II serine/threonine receptors (Massague et al., 1992; Wrana et al., 1994). Approximately twenty different members of the BMP super group have been identified. However, only a subset is able to singly promote osteoinduction, including BMP-2 through -7 and BMP-9 (Abe, 2006; El-Amin et al., 2010; Termaat et al., 2005). Thanks to notable advances in molecular biology since discovering BMP in decalcified bone that induces differentiation of undifferentiated mesenchymal cells into osteoblast (Urist, 1965), human BMP genes have been identified and cloned. Now, recombinant rhBMP-2 are produced and purified from *E.Coli* and mammalian cell lines, but their *in vivo* efficacy is still unclear and needs more study.

To amplify the regeneration efficacy of exogenous BMPs, protein transduction domain (PTD), which delivers passenger protein directly to intracellular space, can be considered. The function of PTDs was discovered from human immunodeficiency virus (HIV)-1 trans-activator of transcription (TAT) protein that enters into cytoplasm in a concentration-dependent manner without going through any receptor system (Frankel and Pabo, 1988; Green and Loewenstein, 1988). HIV-1 TAT fusion protein consists of 86 amino acids, including two essential exons to replicate HIV. Eleven of the amino acids (RKKRRQRRR) are known to regulate unrestricted protein transduction (Schwarze et al., 2000). However, exact transduction mechanisms have not yet been announced.

To acquire successful *in vivo* bone regeneration, the function of carrier and the efficiency of BMP itself are important (Lo et al., 2012). Hyaluronic acid (HA) is one of the major components of the extracellular matrix and is found in all connective tissues of the body. It is a naturally derived, linear, high molecular weight polymer with visco-

elastic properties (Laurent et al., 1995). Due to its visco-elastic properties and biological functions, HA has been used predominantly as a raw material for hydrogel (Prestwich et al., 1998; Vercruysse and Prestwich, 1998). Hydrogel is a hydrophilic, biocompatible material that is able to induce osteoinduction, shows the controlled release of containing drugs, and degrades without foreign body reaction.

Fibrin is a material that can be rapidly invaded, remodeled, and replaced by cell-associated proteolytic activity (Murphy and Gavrilovic, 1999). Although there are conflicts concerning the use of fibrin for *in vivo* bone regeneration application (Patel et al., 2006), fibrin can be recognized as a proper carrier material in bone regeneration (Yang et al., 2010).

Poly ether ether ketone (PEEK) is a biocompatible polymer consisting of calcium phosphate and collagen that can be applied in spinal fusion where mechanical resistance is needed (Boakye et al., 2005; Meisel et al., 2008). Also PEEK's isolating ability of containing materials from external environment helps evaluating regeneration capacity of entrapped material.

For an ideal *in vivo* bone regeneration, regenerative medicine should be released in a controlled manner during scaffold degradation to permit complete repair by the host tissue. In this regard, this study aims to evaluate the *in vivo* bone regeneration efficacy of TAT-BMP-2, which has been found to induce *in vitro* osteogenesis in previous studies by combining with fibrin, hydrogel, and PEEK to search for the most appropriate carrier system.

II. MATERIALS AND METHODS

1. TAT-BMP-2 expression vector

To insert the YGRKKRRQRRR domain of HIV-1 TAT fusion protein into the pRSET (Invitrogen, U.S.A) vector, PCR was done with T7 (5‘-CTCGAGTAATACGACT CACTATAGG-3') primer and 5‘-CATGGTGGATCCACCGCGGCGGCGCTGGCGGCG TTTCTTGCGGCCGTAGCCAAGCTTGGATCC-3' primer. The PCR product and pRSET vector were restricted by one unit of BamHI under 37℃ for two hours. After restriction, they were purified with a HiYield Gel Extraction kit (RBC, Taiwan). The pRSET-TAT vector was constructed by inserting the PCR product into the pRSET vector at the BamHI site. Human BMP-2 cDNA was acquired by PCR method, restricting Saos-2 cell with primers, which have KpnI and HA sequences on C-terminal (5'-GAGATTGGTACCATGGTGGCCGGGACCCGC-3', 5'-AATCTCGGTACCCTAAAGA GCGTAATCTGGAACATCGTATGGGTAGCGACACCCACAACC-3'). Amplified BMP-2 were cloned into the KpnI site of the pREST-TAT vector. To insert N-terminal HA2 peptides (GLFGAIAGFIENGWEGMIDG) of Influenza virus haemagglutinin-2, PCR was done with primers that have the T7 and BamHI sequences (5’-GAGGCCGGATCCACCGTCTATCATTCCCTCCCAACCATTTTCTATGAAACCTGC TATTGCGCCGAATAGGCCACCGCGGCGGCGCTGGCG-3') and pREST-TAT-BMP-2-2-HA2 vectors were made. Additional Xpress and 6x histidine were added to tag the protein, separate them, and purify them by binding with Ni-NAT agarose (Figure 1).

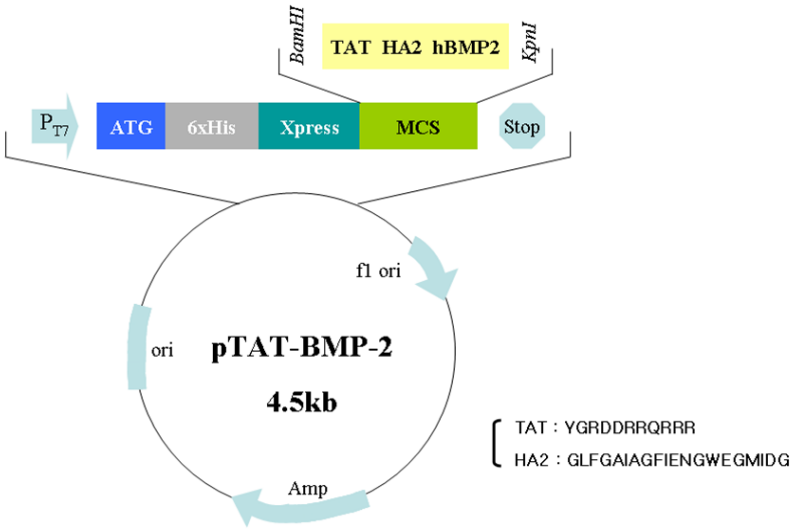


Figure 1. TAT-BMP-2 vector map. HA2 was inserted to avoid entrapment of transduced protein.

2. TAT-BMP-2 protein expression and purification

Recombinant protein was expressed through BL21 (DE3) *Escherichia. coli* (Invitrogen, U.S.A). The pTAT-BMP-2 vector was transformed to bacteria and streaked on a plate that contained 100μg/ml ampicillin. After incubating for over 16 hours, bacteria was collected by scraping and inoculated to 400ml 2 x YT (Q-Biogen, France) LB media, which contains 100μg/ml ampicillin (Sigma, U.S.A). Isopropyl-β-D-thiogalactoside (Sigma, U.S.A) was added to reach a final concentration of 0.4mM when the value of OD600 was between 0.5 and 0.7. After four more hours of cultivation, centrifugation was done for 30 minutes under 5500rpm. Soluble lysis buffer (50mM Tris, pH8.0, 300mM NaCl, 20mM imidazole, pH8.0) 10ml was added to precipitation for resuspension, then sonication (amplitude 90, 60 seconds, two times) was done. Triton X-100 was added to reach final concentration at 0.5 percent, and then centrifugation was done for 20 minutes under 4℃, 12000rpm. To denature the expressed protein, 10ml of inclusion body solution (8M urea, 50mM tris, pH8.0, 100mM NaCl) was added and dissolved for one hour at room temperature, and then centrifugation was done for 15 minutes under 15000rpm. Supernatant reacted to 1ml of nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen, Germany) for two hours under 4℃, which was substituted with binding buffer (8M urea, 50mM tris, pH8.0, 100mM NaCl, 20mM imidazole, pH8.0). Reacted Ni-NTA agarose was washed three times with 10ml of binding buffer and mixed with 500nM, 1mM imidazole binding buffer respectively for 20 minutes under 4℃. Afterward, centrifugation was done for one minute under 1000rpm to purify TAT-BMP-2.

The purified protein was aliquoted 100µl with adding 10 percent glycerol and stored at -70℃. The concentration and purity of the protein was verified through SDS-PAGE gel using bovine serum albumin (BSA) and immunoblot analysis using anti-xpress.

3. Cell culture and cell assay

C3H10T1/2, osteosarcoma cell (OS), and gingival fibroblast (GF) were maintained in Dulbecco's MEM (DMEM) (Gibco, U.S.A) supplemented with 10 percent FBS, under 37˚C, 5 percent CO_2. For the *in vitro* cellular compatibility and viability assay, the fibroblast were kept in contact with the hydrogel, latex, and teflon for 72 hours. The cell proliferation assay was performed with cell counting kit-8 and MTT reagent while the fibroblast were kept in contact with the hydrogel, latex, and teflon.

4. RT-PCR

For monitoring Runt related transcription factor 2 (RUNX2), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression, primer sequences for RT-PCR were: RUNX2 forward, 5-CTCTTCCCAAAGCCAGAGTG-3, RUNX2 reverse 5-CAGCGTC AACACCATCATTC-3, GAPDH forward, 5-TGAAGGTCGGAGTCAACGGATTT-3, GAPDH reverse, 5- CATGTGGGCCATGAGGTCCACCAC-3.

The PCR reactions were performed at 60˚C, 30 cycles for runx2, and 58℃, 28 cycles for gapdh. Amplified products were visualized with a UV illuminator.

5. Immunoblot assay

Western immunoblot analysis was performed to determine the expression of TAT-BMP-2 following the conventional method. Primary antibodies were monoclonal anti-xpress (Invitrogen, U.S.A), and polyclonal anti-Tubulin (Sigma, U.S.A).

6. Acrylation of HA and preparation of hydrogel

Procedures for manufacturing hydrogel were performed following previously reported methods (Kim J. et al., 2007). HA (0.25mmol, based on the repeating unit MW) was dissolved in 40 ml of distilled water and EDC (0.24g, 1.25mmol), HOBT (0.17g, 1.25mmol) and adipic acid dihydrazide (ADH) (2.2g, 12.5mmol) were added to the solution. The EDC mediated the coupling reaction between the carboxyl group of HA and the hydrazide group of ADH with stirring, at room temperature for eight hours. HA–ADH was dialyzed against 100mM NaCl for two and a half days and distilled water for one day, using a dialysis membrane (Molecular weight cut off 14,000) (Spectra/Por; U.S.A). NAS (0.5g, 3mmol) was subsequently added to the HA–ADH solution. The reaction continued, with stirring, at room temperature for 12 hours. HA–ADH–NAS was dialyzed extensively against 100mM NaCl for two and a half days and distilled water for one day. The product was then lyophilized for three days to obtain solid acrylated HA (HA-Ac). The NMR spectra were obtained on a Mercury 200 NMR (200MHz) (Varian, U.S.A). D2O was used as a solvent for all the samples and the reported spectra represented an average of 64

scans. The degree of acrylation was calculated by comparing peaks from the acryl and methyl groups from the HA residue. For gel preparation, acrylated HA was dissolved in a triethanolamine-buffered solution (TEA; 0.3M, pH 8). PEG-SH4 (Molecular weight 10,000), which contains soluble TAT-BMP-2 in planned concentration, was added as a cross-linker with the same molar ratio of the acryl and thiol groups. HA-based hydrogel was formed via a Michael-type addition reaction. The reaction mixture was incubated at 37˚C for gelation. This hydrogel (5 percent weight of HA and PEG-SH4) was used in both *in vitro* and *in vivo* experiments.

7. Animal experiment

Four Beagle dogs (adult male weighting 13.0–14.0 kg) were purchased and fed in separate cages with free access to food and water. This animal study was approved by IACUC of Seoul National University Hospital Biomedical Research Institute. Animals were anesthetized using isoflurane (5 percent induce; 3 percent maintain) and administered subcutaneous atropine 0.05mg/kg, intravenous xylazine 2mg/kg, and zoletil 5mg/kg immediately pre-operatively for pain mitigation. The electrocardiogram was monitored during operation. The surgical site was shaved and disinfected with 10 percent Betadine and subcutaneously injected with 2 percent lidocaine, containing 1:100,000 epinephrine. A linear incision was made along the lateral thigh, and soft tissue and periosteum were reflected. 5mm diameter 6mm depth cylindrical defects were made in both femurs using a trephine bur while irrigating with saline. After extensive saline

irrgation to the defect site, the implant was placed into the defect, and the soft tissue was closed over the defect site using 3-0 vicryl and 3-0 nylon. Each subject was implanted with the following composition: first, fibrin and PEEK; second, fibrin, TAT-BMP-2 1μg, and PEEK; third, hydrogel; and fourth, hydrogel and TAT-BMP-2 2.5μg. Applied PEEK was cylindrical shape with bottom surface opened, 5mm diameter 6mm height 1mm wall thickness and opposing two 1mm diameter holes at the middle of lateral surface. All materials were gelated before insertion. Due to unintended lack of prepared PEEK, only two holes were made on left side of the second subject and no materials were inserted in the middle hole of right femur of the same subject. To avoid post-operative infection, intramuscular cefazolin 10mg/kg was injected once daily for three days. Radiologic examinations were performed to check the healing status of operation site every two weeks with BV Pulsera (Philips, Netherland).

8. Radiological and histological analysis

After allowing the defect to heal for five or six weeks, the animals were sacrificed by intravenous injection of zoletil and chloropotassium. The operative field was harvested and fixed in 10 percent formalin. Micro-computed tomography (microCT) analysis was performed using Skyscan 1076 (Skyscan, Belgium). Acquired images were processed in DICOM format and read by Simplant Crystal (Materialise, Belgium) to observe three-dimensional structure. Bone density and volume were calculated and the spectrum of bone density was acquired by triangle view mode.

The bone pieces without a PEEK scaffold were then decalcified in 10 percent EDTA, pH 7 at room temperature, dehydrated in 70 percent ethanol and embedded in paraffin. The paraffin-embedded sections (5 micrometer) were stained with hematoxylin eosin and viewed using microscopy. Other bone pieces with PEEK scaffolds were embedded in methyl methacrylate, and stained with Villanueva. Digital images from stained sections and microCT were processed with SPOT version 4.5 (Diagnostic instrument Inc., U.S.A) and ImageJ version 1.45p (National Institutes of Health, U.S.A) to calculate the area of regenerated bone.

9. Statistical analysis

Density, area, volume of regenerated bone was obtained and compared each other. Area of the first and second subject was not calculated because their regeneration pattern was too scattered to calibrate. Statistical analysis was processed with SPSS 12.0 (SPSS, U.S.A) using Mann-Whitney test, a nonparametric statistical method. Critical p-value was 0.05.

III. RESULTS

1. Expression of TAT-BMP-2

The TAT-BMP-2 vector was cloned by combining HIV-1 TAT, and HA2 and expressed through the *E.Coli* BL21 (DE3) cell. The expressed protein was almost localized in the inclusion body. They were denatured by 8M urea to improve transduction efficiency and purified by Ni-NTA agarose through tagged histidine. Following the result of Coomassie Brilliant Blue staining, TAT-BMP-2 fusion protein was expressed at 52kDa size (Figure 2).

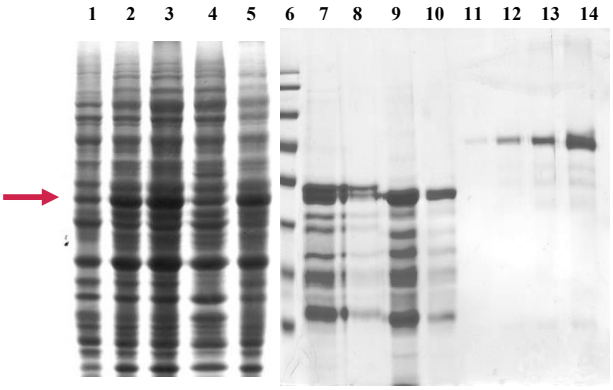


Figure 2. Expression of TAT-BMP-2 fusion protein. The red arrow indicates 52kDa size. Lane1: uninduction, Lane2: nduction, Lane3: soluble lysate, Lane4: supernatant, Lane5: insoluble lysate(inclusion body), Lane6: protein size marker, Lane7: 500mM Immidazole elution, Lane8: 1M Immidazole elution, Lane9: Lane7 dialysis, Lane10: Lane8 dialysis, Lane11: BSA 0.1mg/ml, Lane12: BSA 0.5mg/ml, Lane13: BSA 1mg/ml, Lane14: BSA 2mg/ml.

2. *In vitro* localization and function of TAT-BMP-2

To verify the intracellular transduction, TAT-BMP-2 fusion protein was added in the culturing media of the C3H10T1/2 cell and expressed through the xpress tag. In the immunoblot analysis results, intracellular transduction of TAT-BMP-2 was higher than the negative control (Figure 3A). Also, the RT-PCR results showed that the mRNA expression level of RUNX2, a marker of initial stage osteogenesis, was higher in the TAT-BMP-2 treated cell than in the negative control. This means that immature form TAT-BMP-2 was transduced into intracellular space and then transformed to mature form, which led to osteogenesis (Figure 3B).

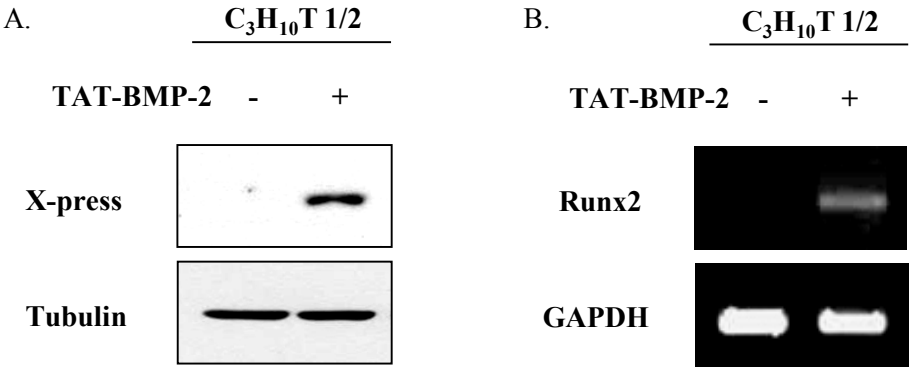


Figure 3. Internalization and function of TAT-BMP-2 fusion protein. A. Immunoblot analysis indicates intracellular transduction of TAT-BMP-2. B. TAT-BMP-2 induced cell shows RUNX2 expression.

3. *In vitro* biological properties of hydrogel

3.1. Compatibility and viability of fibroblast

To evaluate the biocompatibility of hydrogel, the fibroblast were maintained on hydrogel surface and observed by microscope after days three and seven. The fibroblast's survival was observed in the results of both days three and seven (Figure 4A). The fibroblast were maintained on the surface of hydrogel, latex, teflon to test its own viability. Cellular proliferation and differentiation were more favorable in hydrogel than in teflon or latex (Figure 4B).

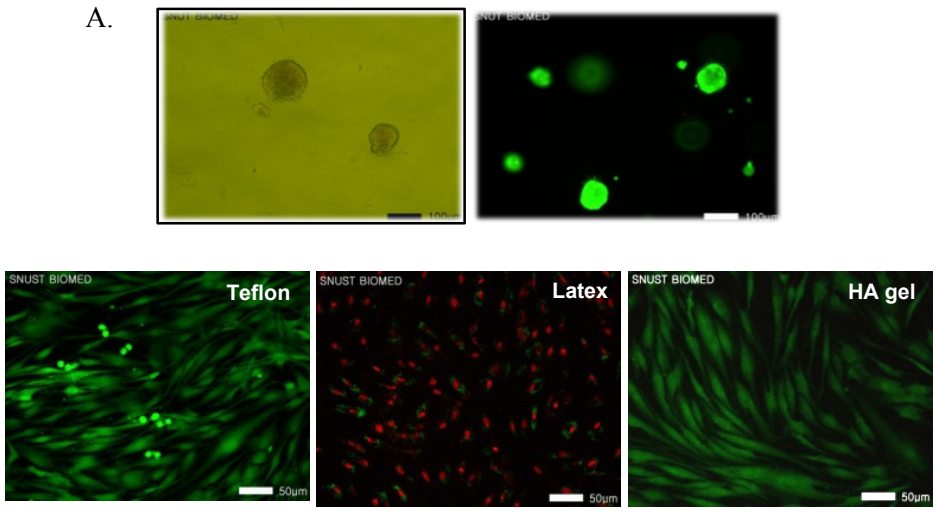


Figure 4. *In vitro* evaluation of compatibility and viability. A. Compatibility of fibroblast on hydrogel. Left panel, day three, right panel, day seven. B. Viability on several polymers after 24 hours maintaining.

3.2. Proliferation of fibroblast on hydrogel

The ability to proliferate was measured by cell counting kit-8 on days one, three, and seven. Absorbance at 450nm was increased as time goes by that the viability of living cells can be sustained on hydrogel (Figure 5A). Cytotoxicity was analyzed by MTT assay and hydrogel showed most favorable viability, in contrast to latex and Teflon (Figure 5B).

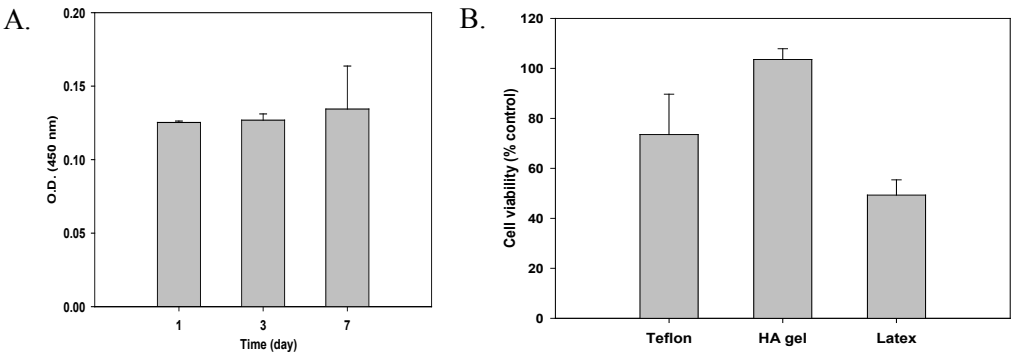


Figure 5. CCK-8 and MTT assay results. A. The result of CCK-8 assay meant activity of dehydrogenase of living cells. B. Cellular viability was observed through MTT assay.

4. Follow up radiograph

Periodic radiographs were taken every two weeks after the animal experiments to evaluate healing status. Despite all defects, all subjects were radioluscent at two weeks. The subject with the TAT-BMP-2 and hydrogel composite showed notably higher radiopaqueness at four weeks after the operation than did the other subjects. The hydrogel-only subject and the TAT-BMP-2 hydrogel composite subject were given radiographs at five weeks again and sacrificed at that time due to their increased radiopaqueness. The fibrin-only and fibrin TAT-BMP-2 composite subjects with PEEK scaffold were given radiographs at the planned six weeks. However, no remarkable radiopaqueness was found (Figure 6).

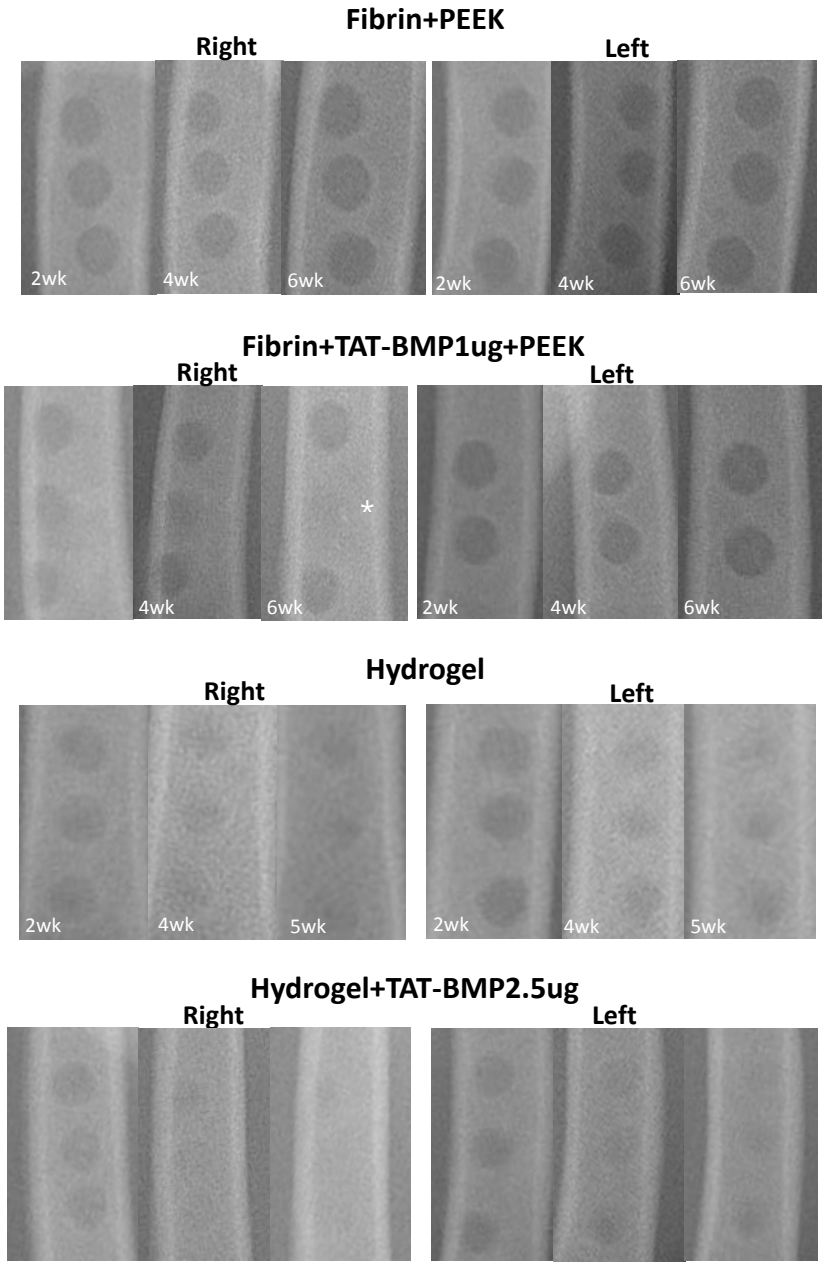


Figure 6. Follow up radiographs. Decreased radioluscency means occurrence of bone regeneration. As there were no materials inserted at astronaut indicated region, the defect was excluded in analysis.

5. MicroCT analysis

After sacrificing all subjects, regions of interest with implanted materials on the femurs were cut and fixed in a 10 percent formalin solution. In microCT images, the subject with the TAT-BMP-2 hydrogel composite showed the highest attenuity at the operation site. Increased attenuation was extended to neighboring marrow space in the same subject. Only the hydrogel applied subject barely showed increased attenuation; the other two subjects showed just weak attenuation in limited marrow space (Figure 7).

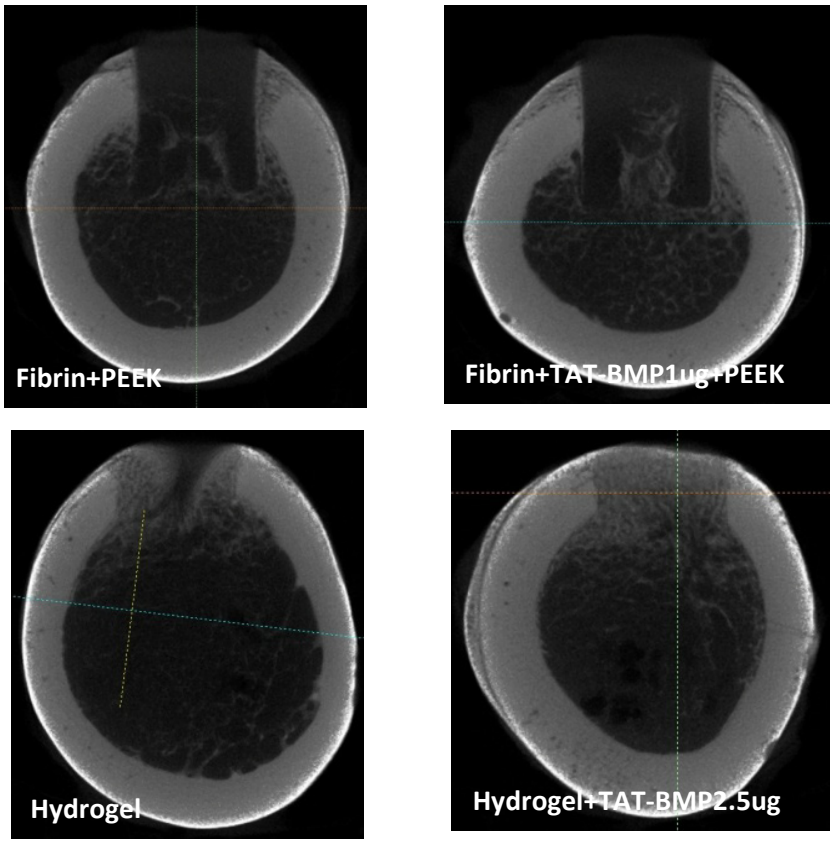


Figure 7. Micro CT images. Images were selected at the middle of defect. Attenuity was mostly increased at TAT-BMP-2 hydrogel composite applied subjects.

5.1. Density of regenerated bone

By adjusting gradient color distribution following tissue density based on the Hounsfield unit, visualization and measuring bone density could be possible. Fat and soft tissue were orange, the inner cortex was green, and the outer cortex was red. The TAT-BMP-2 hydrogel composite applied subject showed the most abundant, almost complete, bone regeneration, which had a similar density with the inner cortex and had only regenerated the outer cortex layer (Figure 8A). Only the hydrogel applied subject was found to have incomplete regeneration at each end side of the defect area. The fibrin-only and fibrin TAT-BMP-2 composite applied subjects showed a small amount of low density hard tissue with no statistical difference (p=0.097). The TAT-BMP-2 hydrogel composite applied subject showed the highest mean bone density on the defect area. Nevertheless, the difference between the hydrogel-only applied subject and the TAT-BMP-2 hydrogel composite applied subjects was 1.6, statistically not significant (p=0.724) (Figure 8B).

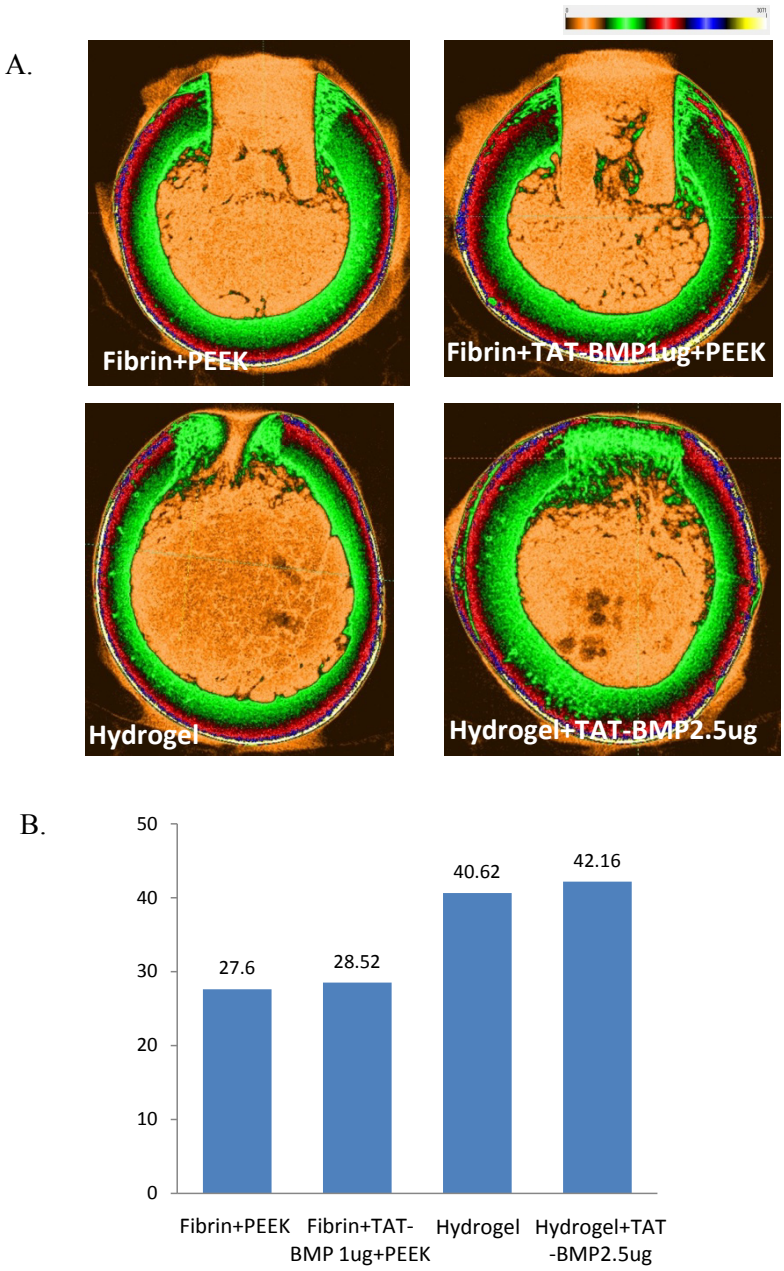


Figure 8. Density analysis of regenerated bone. A. Gradient density view in software. Images were selected at the middle of defect. B. Mean bone density of regenerated bone.

5.2. Area of regenerated bone

The area of regenerated bone on the defect was calculated by selecting the middle slice of the axial image. The total regenerated area was 810.17mm^2 on the TAT-BMP-2 hydrogel composite applied subject, and 504.17mm^2 on the hydrogel-only applied subject. Regenerated area of these two subjects, hydrogel only applied subject and TAT-BMP-2 hydrogel composite applied subject, showed a significant difference (p=0.009). The red labeled outer cortex was found only in the TAT-BMP-2 hydrogel composite applied subject and its area was 82mm^2 (Figure 9).

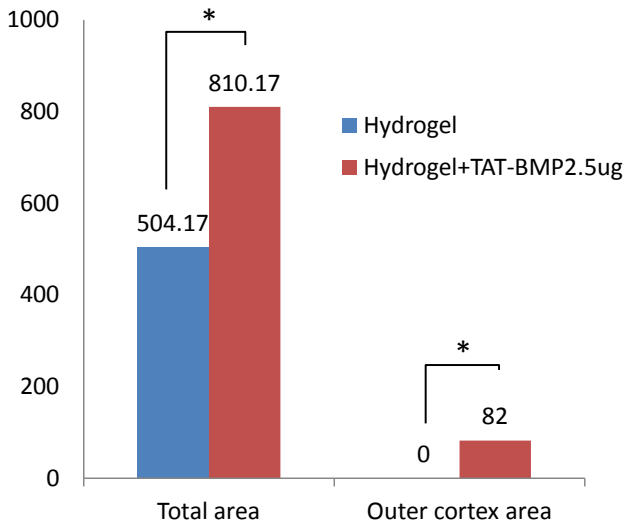
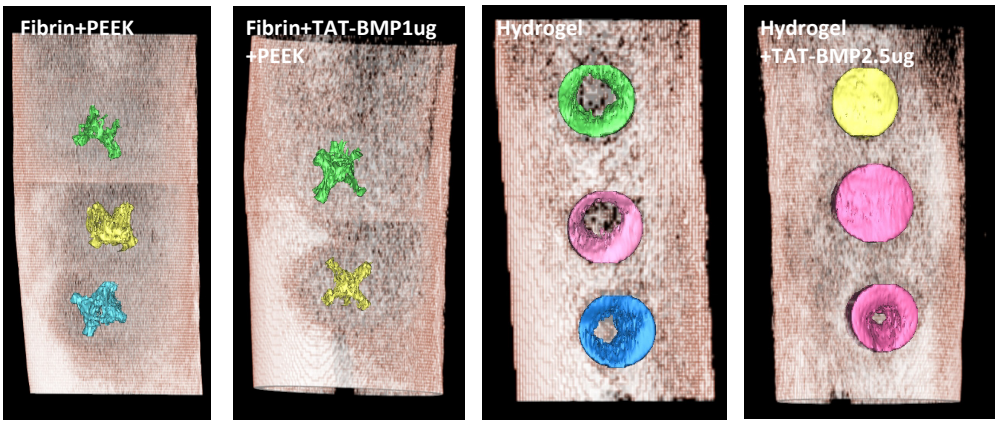


Figure 9. Area analysis of regenerated bone.

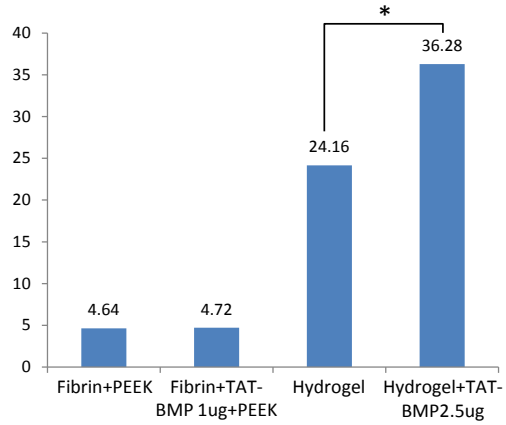
5.3. Volume of regenerated bone

A three-dimensional reconstruction model was made through volume rendering of multiple microCT axial images (Figure 10A). The hydrogel-only applied subject showed an unregenerated and empty appearance at the middle of the defect. In contrast, the TAT-BMP-2 hydrogel composite applied subject showed a completely regenerated or almost blocked appearance on the top surface of the defect. The volume of the regenerated bone was 36.28mm^3 in the TAT-BMP-2 hydrogel composite applied subject, which was 1.5 times larger than the hydrogel-only applied subject (*p=0.009*). The fibrin-only and fibrin TAT-BMP-2 composite with PEEK scaffold showed 4.64mm^3 and 4.72mm^3, respectively (Figure 10B). The regeneration ratio, which confers the volume ratio between regenerated bone and defect, showed that the TAT-BMP-2 hydrogel composite applied subject had the highest regeneration efficiency, 58.44 percent (Figure 10C).

A.



B.



C.

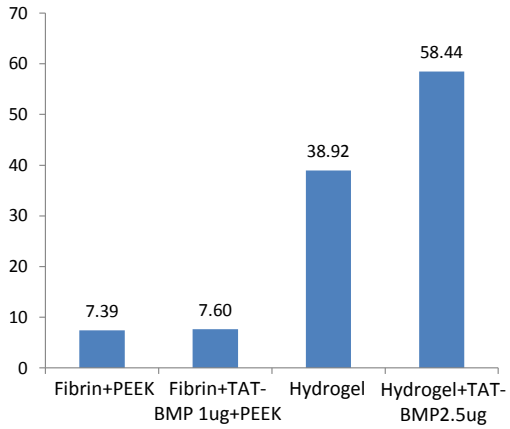


Figure 10. Volume analysis of regenerated bone. A. 3D reconstruction of femur of beagle. B. Volume comparison between subjects. C. Regeneration ratio.

6. Histological analysis

Slides of subjects that did not contain PEEK were obtained through decalcification and hematoxylin-eosin staining. PEEK-containing subjects underwent undecalcification and Villanueva staining. In microscopic images, PEEK-containing subjects showed a little bone regeneration pattern in two regions. One was the open surface of PEEK-facing marrow space and the other was inside small holes at the middle of PEEK. The TAT-BMP-2 hydrogel composite applied subject showed much more abundant regenerated bone than the hydrogel-only applied subject. In addition, the TAT-BMP-2 hydrogel composite applied subject showed regeneration of continuity of the outer cortex and periosteum, an increase of the Harversian system, and osteocyte and periosteal reaction. Regeneration was extended to the adjacent marrow portion and the regenerated bone had a more mature, dense structure (Figure 11).

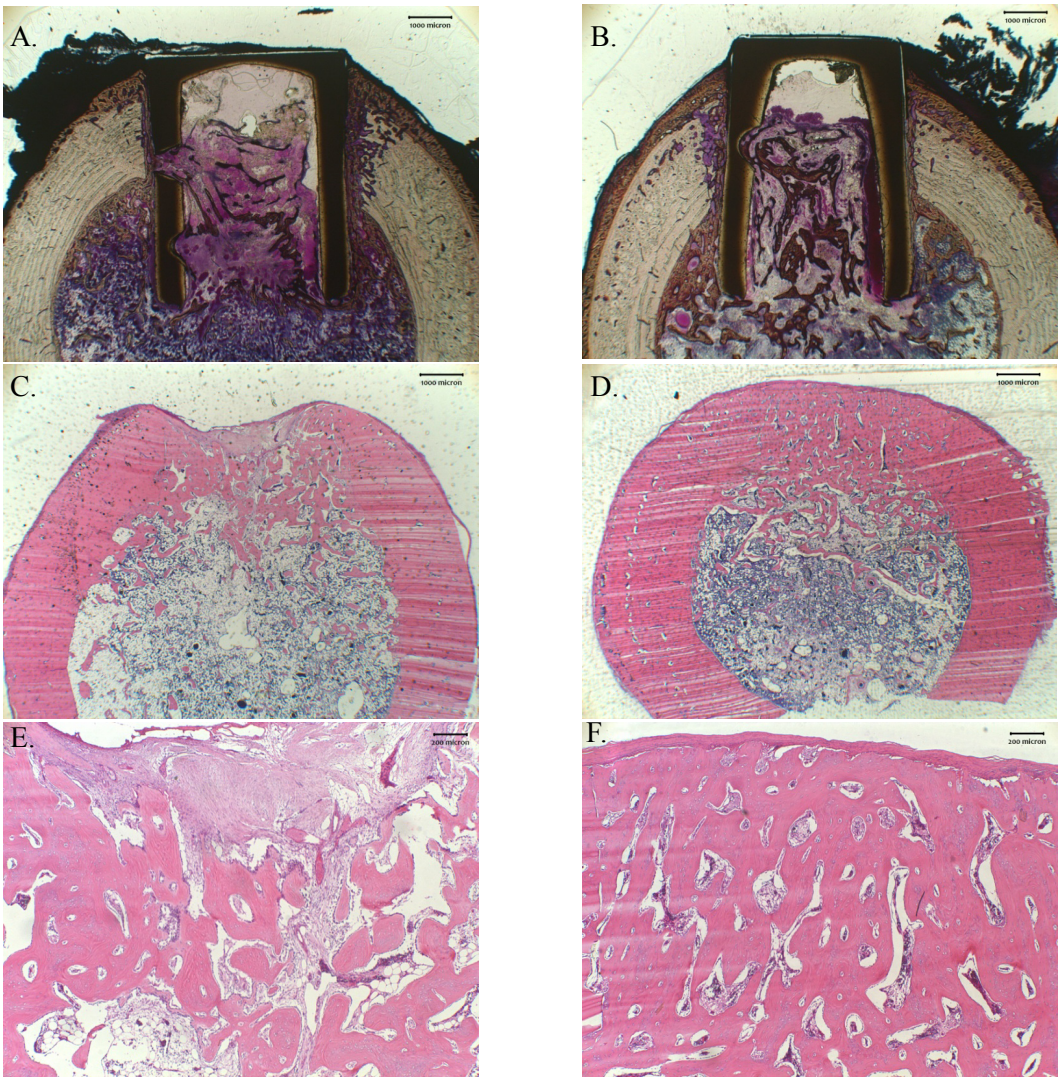


Figure 11. Histlogical analysis of regenerated bone. A. Fibrin with PEEK, x10, B. Fibrin, TAT-BMP-2 1ug with PEEK, x10, C. Hydrogel, x10, D. Hydrogel, TAT-BMP-2 2.5ug, x10, E. Hydrogel, x40, F. Hydrogel, TAT-BMP-2 2.5ug, x40.

IV. DISCUSSION

Regenerating or repairing damaged bone tissue due to various causes is a significant challenge in dentistry, orthopedics, and neurosurgery. Graft materials for defect areas are divided into autograft, allograft, alloplast, and xenograft, with autograft being considered the most promising therapy (Myeroff and Archdeacon, 2011; Nauth et al., 2011). However, shortages such as limited graft accessibility, donor site morbidity, and increased costs are driving research about new materials for bone repair and regeneration (Bishop and Einhorn, 2007; Geiger et al., 2003).

Current biological strategies for bone regeneration with *de novo* synthesized materials are devided in three ways (Rose et al., 2004): gene therapy, the transduction of genes encoding cytokines with osteogenic capacity into cells at repair sites; stem cell therapy, the transplantation of cultured osteogenic cells derived from host bone marrow; and protein therapy, the application of osteoinductive growth factors. Because gene and stem cell based therapy have yet to show definite results (Kimelman et al., 2007), protein therapy can be accepted as the most practical and promising method.

Several studies have tried to regenerate bone in an *in vivo* environment using exogenous BMPs and some have received Food and Drug Administration (FDA) approval for merchandising through pre-clinical examinations. Currently, two rhBMP-based commercial products, INFUSE (rhBMP-2, Medtronics, Minneapolis, MN, U.S.A) and OP-1 (rhBMP-7, Stryker Biotech, Hopkinton, MA, U.S.A) have received FDA approval for several surgical applications. INFUSE uses an absorbable collagen sponge (ACS) and OP-1

uses carboxymethylcellulose sodium as a carrier. In addiontion, Cowell BMP&Boss (Cowell Medi, Korea) delivers rhBMP coated in biphasic calciumphosphate/hydroxy apatite. But, in spite of the splendid developments in rhBMP therapy, it is still obscure and questionable in two ways: first, there have been problems in combination with current carriers, and second, the supraphysiological dosage of rhBMP for desired ostoinductive effect. Currently, rhBMP-2 is supplied with an ACS carrier or a biphasic calciumphosphate/hydroxy apatite in which rhBMP-2 is coated. Although the carrier's ability to release biologic factors in a controlled manner is one of the most important parts of tissue engineering, current carriers are still insufficient in this aspect and cause a short *in vivo* half life for rhBMP-2. In fact, Hollinger *et al.* indicated that due to initial burst release, less than 5 percent of rhBMP remains within the collagen sponge at two weeks *in vivo* (Hollinger et al., 1998). Specifically, since the collagen sponge lacks mechanical integrity, the local concentration of rhBMP-2 can increase to undesirably high levels as the sponge is compressed by overlying muscles and other tissue. Also, since rhBMP-2 is physically entrapped in the collagen matrices and depend on matrix degradation for release, their release kinetics are unpredictable and difficult to control (Tan et al., 2007). Because a lack of controlled releasing triggers the second problem, rhBMP-2's minimal concentration for *in vivo* bone regeneration increases the chance that clinical complications such as soft tissue edema, erythema, local inflammation, heterotropic ossification, immune response, or osteoclastic activity might arise by supraphysiologic concentration (Lo et al., 2012). Also, rhBMP-2 from CHO cells or bacteria are lacking in post-translational modification, their biologic effect is depressed or insufficient (Itoh et al., 1999). So the actual dosage for clinical application (milligram

level) became several million times more elevated than the endogenous BMP level (nanogram level), which led to a considerable rise in cost (Haidar et al., 2009; Luginbuehl et al., 2004).

To solve these current problems, a previous study manufactured an intracellular BMP delivery system using PTD (Kim NH., 2007). Kim purified TAT-BMP-2 from *E. Coli*., denatured it to improve transduction efficiency, and demonstrated *in vitro* osteogenesis inducibility. To prove TAT-BMP-2's ability *in vivo*, its soluble property causing rapid clearance should be compensated with biomaterial that are able to retain and sequester biologic factors for elevating efficacy and lowering protein dosage.

Fibrin and hydrogel were selected as carriers for this experiment because they are injectable in clinical applications and natural polymers that guarantee biocompatibility degrading without foreign body reactions. However, as fibrin degrades more rapidly than hydrogel by proteolytic activity, fibrin is hard to acquire controlled releasing pharmacokinetics. In addition, hydrogel can be manufactured by several methods that results in different specifications of polymerization, gelation, and degradation (Toh et al., 2012). Hydrogels also have characteristics to induce vasculogenesis and angiogenesis, which are beneficial in osteogenesis (Hanjaya-Putra et al., 2012).

A PEEK scaffold was used in this experiment to isolate containing materials from external environment in evaluating osteogenesis. The two subjects with PEEK showed a little amount of regeneration from the marrow space and the small holes in the middle of the PEEK body. Though TAT-BMP-2 fibrin composite induced a little more abundant matured regeneration, no significant differences were found between fibrin-only applied subject. Also, the quantity and quality of regenerated bone in PEEK applied subjects were

inferior than the subjects which did not contain PEEK. Hence, even if the TAT-BMP-2 fibrin composite induced superior bone regeneration than the fibrin-only applied subject under isolated condition by PEEK, the inferiority of their entire regeneration efficacy than without PEEK applied subjects are caused not only the low porosity of the PEEK design but also inadequacy of fibrin as a slow degrading-releasing carrier..

Though the hydrogel-only application induced partial bone regeneration, the TAT-BMP-2 hydrogel composite showed more effective regeneration than the hydrogel alone. The TAT-BMP-2 hydrogel composite induced one and a half times the regeneration in area, volume analysis, and restoration of continuity of the outer cortex with favorable bone density. Also, the quantity and quality of regenerated bone were superior to that of the hydrogel-only applied subject. This is practically originated by TAT-BMP-2's *in vivo* osteogenesis inducibility based on proper chemical binding between TAT-BMP-2 and the hydrogel carrier that makes controlled BMP release during slow carrier degradation.

Besides the TAT-BMP-2 hydrogel composite's active bone regeneration, this combination suggested another advantage in practical trials. The concentration of TAT-BMP-2 in this experiment was in micrograms, which is a much lower concentration than that of currently merchandising rhBMP-2 (mg/ml level). As previous *in vitro* study demonstrated that the minimal concentration for BMP-2 secretion was 1μg/ml (Kim NH., 2007), the quantity of TAT-BMP-2 for isolated condition was set at 1μg and 2.5μg for TAT-BMP-2 hydrogel composite considering diffusion. Consequently, TAT-BMP-2 indicated a new way to overcome several complications by supraphysiologic concentration of rhBMP-2.

Though TAT-BMP-2 is a still an incomplete molecule that needs to be improved, its bone regenerating ability is demonstrated both *in vitro* and *in vivo*. To continue making advances, and to ultimately get approval for clinical use, more advanced animal studies in special functional surgery with lower dosages are needed in the near future.

V. CONCLUSION

1. TAT-BMP-2 fibrin composite induced superior bone regeneration than the fibrin-only applied subject under isolated condition by PEEK, but statistically not significant.

2. Hydrogel-only application induced partial bone regeneration.

3. TAT-BMP-2 hydrogel composite caused bone regeneration superior to that of hydrogel-only application in area and volume analysis ($p<0.05$).

4. TAT-BMP-2 hydrogel composite only induced regeneration of continuity of the outer cortex and showed a more mature, dense structure than hydrogel-only application.

5. TAT-BMP-2 hydrogel composite decreased dosage for *in vivo* bone regeneration by 1/1000 of the conventional dosage of rhBMP-2.

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ABSTRACT (IN KOREAN)

TAT-BMP-2와 Hydrogel 복합체의 생체 내 골 재생 능력 평가

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노령화 사회로 진행됨에 따라 치조골 소실 및 골다공증 환자가 증가하고 있으며, 골 재생의 필요성은 점차 증가하고 있으나 현재까지 명확한 해결책은 부족한 실정이다. 외인성 골 형성 유도물질이 효과적으로 작용하기 위해서는 충분한 생체활성을 지녀야 하며 재료의 방출을 조절할 수 있는 전달체와 생물학적, 화학적, 기계적 조화를 이루어야 한다. 생물학적 골재생을 위해 재조합 골형성단백질 (rhBMP-2)가 임상적으로 시도되고 있으나 낮은 효율성을 보이는 것으로 보고되고 있다.

본 연구에 사용된 TAT-BMP-2는 기존의 rhBMP-2와는 다른 비활성 폴리펩타이드 형태로, rhBMP-2가 BMP 수용체와 결합하여 활성을 나타내는 활성 단백질인 것과는 달리, BMP 수용체와는 무관하게 세포 내로 이송되는 특징을 지니고 있다. 하이드로겔은 결합조직 세포외기질의 주요 성분인 하이알루론산을 원료로 합성되며 생체적합성이 우수하고 생흡수와 동시에 약물 방출 속도를 조절할 수 있는 특성을 지니고 있다. 본 연구는 in vitro에서 합성한 TAT-BMP-2를 피브린/Polyether ether ketone(PEEK), 하이드로겔 등의 전달체와 조합하여 비글견 대퇴골에서의 골 재생 효과를 방사선학적, 조직학적으로 관찰하고자 하였으며, 다음과 같은 결과를 얻었다.

1. PEEK에 의해 고립된 환경에서 TAT-BMP-2 피브린 복합체는 피브린만 적용한 경우보다 양호한 골 재생을 보이지만 유의미한 차이를 나타내지는 않는다.
2. 하이드로겔만 적용한 경우에도 부분적인 골 재생이 관찰된다.
3. 하이드로겔 단독으로 사용한 경우보다 TAT-BMP-2와 하이드로겔 복합체를 사용하였을 때, 생체 내 골 재생이 면적, 부피 면에서 모두 150% 가량 우수하였다.
4. TAT-BMP-2 하이드로겔 복합체를 사용했을 때에만 조직학적으로 외피질골의 연속성이 회복되었으며 하이드로겔만 사용한 경우보다 성숙, 치밀한 구조가 관찰된다.
5. TAT-BMP-2 하이드로겔 복합체는 생체에서 골 재생을 위해 요구되는 BMP의 농도를 rhBMP-2의 1/1000 수준으로 낮출 수 있다.

이상의 결과에서 TAT-BMP-2와 하이드로겔 복합체는 생체 내에서 효과적인 골 재생 효과를 유도하는 것을 알 수 있었으며, 이러한 효과는 비활성 폴리펩타이드 형태의 TAT-BMP-2에 의한 고효율의 단백질 형성 및 하이드로겔에 의한 단백질의 조절 분비에 의한 것으로 사료된다.

핵심어 : 단백질 전달영역, HIV-1 TAT, 골형성단백질, 하이드로겔